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Task Distribution between Acetate and Acetoin Pathways To Prolong Growth in *Lactococcus lactis* under Respiration Conditions

Bénédicte Cesselin,[a] Christel Garrigues,[b] Martin B. Pedersen,[b]* Célia Roussel,[a] Alexandra Gruss,[a] Philippe Gaudu[a]

[a]Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France
[b]CED-Discovery, Chr. Hansen A/S, Hørsholm, Denmark

ABSTRACT *Lactococcus lactis* is the main bacterium used for food fermentation and is a candidate for probiotic development. In addition to fermentation growth, supplementation with heme under aerobic conditions activates a cytochrome oxidase, which promotes respiration metabolism. In contrast to fermentation, in which cells consume energy to produce mainly lactic acid, respiration metabolism dramatically changes energy metabolism, such that massive amounts of acetic acid and acetoin are produced at the expense of lactic acid. Our goal was to investigate the metabolic changes that correlate with significantly improved growth and survival during respiration growth. Using transcriptional time course analyses, mutational analyses, and promoter-reporter fusions, we uncover two main pathways that can explain the robust growth and stability of respiration cultures. First, the acetate pathway contributes to biomass yield in respiration without affecting medium pH. Second, the acetoin pathway allows cells to cope with internal acidification, which directly affects cell density and survival in stationary phase. Our results suggest that manipulation of these pathways will lead to fine-tuning respiration growth, with improved yield and stability.

IMPORTANCE *Lactococcus lactis* is used in food and biotechnology industries for its capacity to produce lactic acid, aroma, and proteins. This species grows by fermentation or by an aerobic respiration metabolism when heme is added. Whereas fermentation leads mostly to lactic acid production, respiration produces acetate and acetoin. Respiration growth leads to greatly improved bacterial growth and survival. Our study aims at deciphering mechanisms of respiration metabolism that have a major impact on bacterial physiology. Our results showed that two metabolic pathways (acetate and acetoin) are key elements of respiration. The acetate pathway contributes to biomass yield. The acetoin pathway is needed for pH homeostasis, which affects metabolic activities and bacterial viability in stationary phase. This study clarifies key metabolic elements that are required to maintain the growth advantage conferred by respiration metabolism and has potential uses in strain optimization for industrial and biomedical applications.

KEYWORDS *Lactococcus lactis*, respiration, acetate, acetoin, biomass yield, survival, lactic acid bacteria, transcriptome

Lactococcus lactis is a Gram-positive bacterium widely used in industrial dairy fermentations for its capacity to produce mostly lactic acid (hallmark of fermentation) and to degrade milk casein (1). Due to its technological importance, *L. lactis* energy metabolism has been intensively studied, for instance, to improve the acidification rate by fermentation. In *L. lactis*, acidification depends mainly on one lactate dehydrogenase (encoded by *ldh* of the *las* operon, although other *ldh* genes are present [2]), which

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Address correspondence to Philippe Gaudu, philippe.gaudu@jouy.inra.fr.
* Present address: Martin B. Pedersen, R&D, Sacco SRL, Cadorago, Italy.

catalyzes reduction of pyruvate to lactate. Its enzymatic activity is governed at the transcriptional and posttranslational levels: (i) the carbon catabolite regulator, CcpA, upregulates *ldh*; (ii) the intracellular NADH/NAD ratio and/or fructose-1,6-diphosphate pools modulate Ldh activity *in vitro* (3). Moreover, Ldh phosphorylation may also affect its activity (4). When growth conditions, like aeration and galactose instead of glucose, lead to altered metabolite pools, *L. lactis* strain MG1363 reroutes pyruvic acid from lactic acid production toward a mixture of end products (lactic acid, acetic acid, acetoin, 2,3-butanediol, and ethanol) in a process designated mixed-acid fermentation (MAF). In MAF, lactic acid remains the major end product of sugar degradation. However, we found that no lactic acid is produced under aerobic conditions when heme is supplied, as *L. lactis* strain MG1363 (or industrial CHCC2862) switches from fermentative metabolism to aerobic respiration and produces predominantly acetoin and acetic acid (5–8). Remarkably, this profound metabolic change is accompanied by extended growth and increased long-term survival once cells reached stationary phase (5). To date, the link between the metabolic changes and these phenotypes remains elusive. Previous transcriptome analyses performed in mid-exponential-phase cells did not reveal respiration-specific changes in expression of genes related to carbon metabolism (8). We proposed that the respiratory chain competed with Ldh toward NADH to explain the decrease in lactic acid production. Moreover, proton extrusion activity in the respiratory chain was reported to relieve the ATPase by pumping protons out of cells (9). Compared to that during fermentation, the ATP pool increased during respiration, making it more available for growth (10). An increased ATP pool might also come from acetate pathway activity via acetate kinase, producing ATP. Thus, acetate and acetoin pathways might be more directly implicated in respiration phenotypes than just a sink to eliminate pyruvate for maintaining glycolytic activity. Here, we pursue the question of *L. lactis* metabolic changes by examining cells late in respiration growth. Using a transcriptome approach, we found that several genes related to pyruvate catabolism, like those involved in acetate and acetoin production, were induced late in growth. Mutational analyses of constituents of these pathways have led us to identify their specific contributions to respiratory growth of *L. lactis*, resulting in enhanced biomass production and survival.

RESULTS

Transcriptional changes associated with a metabolic shift during aerobic respiration. Unlike fermentation metabolism, which generates lactic acid as a main by-product of carbohydrate catabolism, respiration leads to a strong increase of acetoin and acetic acid at the expense of lactic acid (Fig. 1A) (5, 8). As in our previous transcriptome (and proteome) analyses performed on mid-exponential-phase cells, expression of carbon metabolism genes was little affected, leading us to propose that the metabolic shift was mainly based on metabolic pool shifts or changes in gene expression that occur later in growth (8, 11, 12). To confirm the latter hypothesis, we performed transcriptomic analysis of the industrial strain CHCC2862 grown with lactose as the energy source, as was done previously (8). Medium was supplemented with β-glycerophosphate buffer to maintain pH around 6 to limit acid stress (8). This supplementation had no visible effect on growth rate or final biomass yield (see Fig. S1 in the supplemental material). RNA pools purified from cells harvested at the entry of stationary phase (EnST) and early stationary phase (EaST; about 40 min after EnST) were compared to RNA from cells harvested at mid-growth phase (optical density at 600 nm [OD_{600}] of 2.5 was used as a reference) (Fig. 1B). While gene expression undergoes massive changes between exponential and stationary phases, we focused on genes linked to carbon metabolism (sugar uptake and degradation) and transcriptional regulators (Table 1). Several genes encoding transporters dedicated to sugar uptake (maltose, glycerol, fructose, and cellobiose) were upregulated late in growth, with the exception of the glucose-mannose transporter (*ptnABCD*; PTS^{Man}), which was downregulated. In contrast to most genes involved in glycolytic activity, major expression changes were associated with pyruvate catabolism, particularly under the EaST condi-

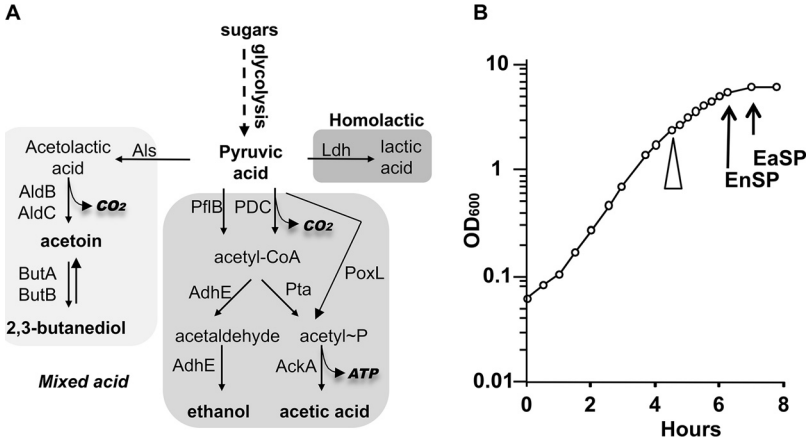

FIG 1 Schematic representation of pyruvate catabolism and growth of *L. lactis* strain CHCC2862. (A) Under homolactic fermentation (dark gray), pyruvate was mainly degraded by lactate dehydrogenase (Ldh). According to environmental conditions, pyruvate is converted to other end products (lighter gray panels; products in boldface). Enzymes for acetoin and 2,3-butanediol production are the following: acetolactate synthase (Als), acetolactate decarboxylase (AldB or AldC), and acetoin-butanediol reductase (ButA or ButB). Enzymes producing acetate and ethanol are pyruvate formate lyase (PflB), pyruvate dehydrogenase complex (PDC), pyruvate oxidase (PoxL), phosphotransacetylase (Pta), acetate kinase (AckA), and acetaldehyde/alcohol dehydrogenase (AdhE). The mixed-acid fermentation (MAF) is formed by production of acetoin, 2,3-butanediol, ethanol, and acetic acid. Production of CO_2 and ATP is represented in boldface, shaded italics. (B) Growth curve of *L. lactis* strain CHCC2862 on M17 lactose. Arrows indicate samples harvested for RNA preparation and transcriptomic analyses, as discussed in this study. The white arrow corresponds to the reference (see Results). EnSP, entry to stationary phase; EaSP, early stationary phase.

tion. Several MAF-related genes were highly induced, including those (i) in the acetate pathway, *pdhABCD* (~4-fold), *poxL* (~15-fold), and, unexpectedly, *pflB* (~16-fold), which encodes an oxygen-sensitive enzyme (13); and (ii) in the acetoin pathway, *als* (~5-fold), *aldB* (~4-fold), *butA* (~95-fold), and *butB* (~80-fold) (Table 1 and Fig. 1A). In contrast, *ldh* expression decreased modestly (~3-fold). These observations were confirmed by transcriptional fusions for some genes in MG1363, the *L. lactis* model (Fig. 2). β-Galactosidase activities indicated a higher gene expression in cells harvested in late exponential phase than in exponential phase. Our results indicated that both of these strains displayed similar behavior under respiration growth conditions, as expected.

Expression of 15 transcriptional regulators was altered in EaST and EnST. One is a two-component system response regulator, LlrA. LacR controls genes of lactose uptake and degradation (or modification), RcfB is related to pH homeostasis (14), and GlnR is implicated in nitrogen metabolism (15). The 11 other regulators have unknown functions.

In conclusion, our analyses indicate a major shift in expression of respiring *L. lactis* reaching stationary phase.

The acetate pathway contributes to growth yield. To analyze the role of the acetate pathway on growth, we compared biomass yield and profiles of glucose degradation end products in a set of mutants growing under fermentation (static and aerated conditions) and respiration (heme in aerated medium). Genes involved in pyruvate degradation were selectively inactivated; they encode the aerobic pyruvate dehydrogenase complex (PDC; *pdhA* of the *pdhABCD* operon) and anaerobic pyruvate formate lyase (Pfl; *pflB*) (Fig. 1A and Table 1). *ldh* was not tested, as almost no lactic acid is detected in MG1363 under respiration growth conditions. *poxL* was also omitted; PoxL catalyzes oxidation of pyruvate into acetyl-phosphate and hydrogen peroxide (H_2O_2), but in the MG1363 strain, amounts of H_2O_2 were almost undetectable (11), indicating that activity in respiration metabolism was marginal in MG1363 compared to that of *L. lactis* CHCC2862.

pflB disruption impaired full biomass yield under low oxygen tension (static growth) (Table 2). This was expected, as this enzyme is essential for acetyl-coenzyme A pro-

TABLE 1 Expression of genes linked to carbon metabolism under respiration-permissive growth conditions[a]

Gene and category	Expression at: EnST	Expression at: EaST	KEGG annotation
Pyruvate catabolism			
Homolactic pathway			
ldh	1.1	−2.7	L-Lactate dehydrogenase
ldhX	−2.4	−2.8	L-Lactate dehydrogenase
MAF pathway			
Acetate pathway			
pflB	6.0	16.3	Pyruvate-formate lyase
pdhA	1.2	4.4	Subunit of pyruvate dehydrogenase
pdhB	1.4	4.1	Subunit of pyruvate dehydrogenase
pdhC	1.2	3.8	Subunit of pyruvate dehydrogenase
pdhD	−2.2	1.9	Subunit of pyruvate dehydrogenase
pta	−1.9	−1.2	Phosphate acetyltransferase
poxL	7.6	15.5	Pyruvate oxidase
ackA1	2.2	−1.5	Acetate kinase
ackA2	1.6	2.4	Acetate kinase
adhE	−2.3	−40.5	Alcohol dehydrogenase/acetaldehyde dehydrogenase
Acetoin pathway			
als	1.3	5.3	Acetolactate synthase large subunit
aldB	1.9	3.7	Alpha-acetolactate decarboxylase
aldC	−1.5	−3.2	Alpha-acetolactate decarboxylase
butA	1.0	96.3	Acetoin reductase
butB	1.8	78.0	2,3-Butanediol dehydrogenase
Sugar transporter			
celB	1.0	22.3	Cellobiose-specific PTS system IIC component
fruA	1.2	37.8	Fructose-specific PTS system enzyme IIBC component
glpF1	1.0	31.9	Glycerol uptake facilitator
glpF2	−2.1	26.1	Glycerol uptake facilitator
malE	−1.0	96.6	Maltose ABC transporter substrate binding protein
malF	1.0	44.1	Maltose ABC transporter permease protein
malG	1.1	79.9	Maltose ABC transporter permease protein
ptbA	−2.2	9.2	Beta-glucoside-specific PTS system IIABC component
ptcA	−2.4	14.5	Cellobiose-specific PTS system IIA component
ptcB	−1.1	83.1	Cellobiose-specific PTS system IIB component
ptnAB	−7.2	−16.8	Mannose-specific PTS system component IIAB
ptnC	−4.9	−12.5	Mannose-specific PTS system component IIC
ptnD	−2.0	−1.2	Mannose-specific PTS system component IID
yedE	−2.9	3.3	PTS system, beta-glucoside-specific IIA component
yedF	−4.9	3.2	Beta-glucoside-specific PTS system IIBC component
ypcH	−1.1	13.4	Sugar ABC transporter permease protein
ypdA	−1.8	10.5	Sugar ABC transporter substrate binding protein
Carbon catabolism/modification			
enoB	2.0	5.8	2-Phosphoglycerate dehydratase
bglH	−2.2	8.6	Beta-glucosidase
bglS	−1.1	9.3	Beta-glucosidase A
dhaI	−1.3	24.9	Dihydroxyacetone kinase
dhaM	1.2	17.2	Dihydroxyacetone kinase
galE	−1.0	94.0	UDP-glucose 4-epimerase
glpD	−4.3	18.2	Alpha-glycerophosphate oxidase
malQ	−1.0	42.9	4-Alpha-glucanotransferase
pgmB	−1.2	4.5	Beta-phosphoglucomutase
Regulators			
aldR	2.8	4.9	Transcriptional regulator
glnR	2.1	−14.5	Glutamine synthetase repressor
gntR	3.4	3.6	Transcription regulator
lacR	2.2	22.9	Lactose transport regulator
llrA	1.5	6.7	Two-component system regulator
rcfB	13.4	7.7	Transcriptional regulator
rgrA	1.3	7.7	Transcriptional regulator
rmaG	−5.0	−3.5	Transcriptional regulator
rmaH	−1.4	−6.8	Transcriptional regulator
rmaJ	−1.3	−3.5	Transcriptional regulator
rmeD	−2.5	85.1	Transcriptional regulator

(Continued on next page)

TABLE 1 (Continued)

Gene and category	Expression at: EnST	EaST	KEGG annotation
ydcG	3.7	3.2	Transcriptional regulator
yebF	−1.6	−3.3	Transcriptional regulator
yecA	−1.4	6.3	Transcriptional regulator
yxcB	3.1	−1.1	Transcriptional regulator

[a]Expression levels in bacteria harvested at the entry (EnST) and early stationary (EaST; about 40 min after EnST) phases were compared to those in mid-growth-phase cells. Ratios were calculated based on reference transcriptome at an OD_{600} of 2.5 (see Materials and Methods). Presented are genes linked to pyruvic acid catabolism, carbon metabolism/modification, sugar transporters, and regulators (regulators which expression differed by >3-fold). Complete transcriptome results are available at NCBI GEO under accession number GSE56776.

duction in anaerobiosis. The mutant displayed no phenotype under aeration, as it is functionally replaced by PDC (16, 17). Unexpectedly, the *pflB* mutant was defective for aerobic respiration (Table 2). The OD_{600} of the mutant was reproducibly lower than that of the wild-type strain (WT) (4.6 for the mutant and 5.2 for the WT; $n = 5$). To determine whether this difference was significant, we isolated a revertant *pflB* strain (see Materials and Methods) that displayed OD_{600} and pH values close to those of the WT (Table 2), confirming that Pfl activity was restored under static fermentation and aerobic respiration growth. Further, we compared end products of glucose degradation produced by *pflB* and the WT strain (Table 2). Under static fermentation, the *pflB* deletion led to a 35% increase in lactate production (~57 compared to ~42 mM/OD_{600} for mutant and WT, respectively) and trace amounts of acetoin. Under aeration, the *pflB* mutation led to a modest increase of acetoin. Heme addition in aeration led to decreased acetate levels (~4.5 versus ~5.8 mM/OD_{600} for mutant and WT, respectively). These measurements suggest that Pfl activity contributes to aerobic respiration growth, although its role was modest. Further, we constructed a *pdhA* mutant to determine whether Pfl was sufficient to support respiration growth. The *pdhA* mutant was constructed in cells growing in the presence of acetate, as described by Henriksen and Nilsson (17), but failed to grow in the presence of oxygen. This result indicates that Pfl activity is not sufficient to replace the PDC during aerobic respiration growth.

The observed minor effects of Pfl and essentiality of PDC on aerobiosis did not lead to a clear view of the role of the acetate pathway in respiration growth. We decided to

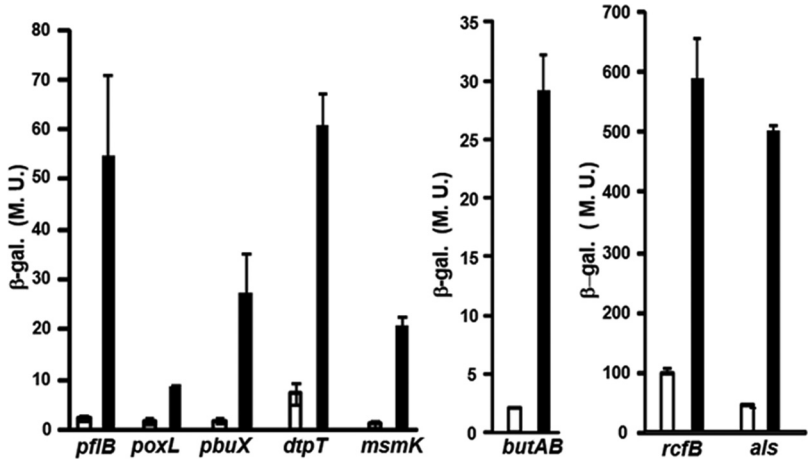

FIG 2 Gene expression under respiration growth conditions. Expression levels of genes were analyzed via transcriptional fusion. Cells were grown in M17 with 1% glucose under respiration growth conditions. Samples were harvested in exponential (OD_{600} of 0.5, white bars) and stationary (overnight, black bars) phases for β-galactosidase determinations. Specific activities are expressed in Miller units (M. U.) ± standard deviations and represent the means from at least three independent experiments. For *pflB*, *poxL*, *pbuX*, *dtpT*, and *msmK*, *lacLM* genes were integrated downstream of the gene in the *L. lactis* MG1363 chromosome, while in other constructions, promoters were cloned in the replicative plasmid pAK80.

TABLE 2 Growth and end products of glucose degradation in *L. lactis* strains[a]

Strain and condition	OD_{600}	pH	Level of[b]: Lactate	Acetate	Acetoin
WT					
S	2.8 ± 0.1	4.4 ± 0.1	42.4 ± 3.2	0.6 ± 0.15	LD
A	2.8 ± 0.2	4.65 ± 0.1	34.6 ± 2.9	3.8 ± 0.6	1.2 ± 0.7
R	5.2 ± 0.2	6.2 ± 0.2	0.2 ± 0.2	5.8 ± 0.6	6.6 ± 1.3
pflB mutant					
S	1.9 ± 0.2	4.5 ± 0.2	57.1 ± 4.2	0.7 ± 0.4	0.15
A	2.7 ± 0.1	4.6 ± 0.1	32.25 ± 2.4	3.9 ± 0.7	2.1 ± 0.1
R	4.6 ± 0.1	6.2 ± 0.1	0.2	4.5 ± 0.2	6.7 ± 0.2
pflB revertant					
S	2.8 ± 0.1	4.45 ± 0.1	ND	ND	ND
A	2.9 ± 0.1	4.7 ± 0.1	ND	ND	ND
R	5.1 ± 0.2	6.2 ± 0.2	ND	ND	ND
pta mutant					
S	2.6 ± 0.1	4.4 ± 0.2	41.5 ± 3.1	0.4 ± 0.1	LD
A	2.85 ± 0.3	4.8 ± 0.2	31.5 ± 0.2	0.5 ± 0.0	3 ± 0.7
R	3.7 ± 0.2	6.3 ± 0.2	8.5 ± 2.6	0.3 ± 0.0	8.6 ± 2
als mutant					
S	2.7 ± 0.1	4.45 ± 0.2	43.8 ± 5.3	0.6 ± 0.1	LD
A	2.6 ± 0.2	4.5 ± 0.2	39 ± 4.1	3.9 ± 0.3	LD
R	3.5 ± 0.2	4.8 ± 0.2	16.3 ± 2.8	7.35 ± 1.3	0.6 ± 0.3
als mutant/*als*$^+$ complemented					
S	2.7 ± 0.2	4.5 ± 0.1	45.3 ± 10.2	0.6 ± 0.1	LD
A	2.6 ± 0.1	4.6 ± 0.1	44.85 ± 7	4.35 ± 0.7	0.5 ± 0.1
R	4.1 ± 0.4	5.55 ± 0.1	10.3 ± 4.1	9.1 ± 1.1	5.4 ± 1.7
als mutant + NaOH					
R	4.7 ± 0.1	6.2 ± 0.1	ND	ND	ND

[a]S, static; A, aeration; R, respiration.
[b]Metabolite levels were determined from overnight culture and expressed in mM/OD_{600}. LD, limit of detection; ND, not determined. Means of data represent averages from at least four independent experiments.

test the effect of inactivating *pta*, encoding the phosphotransacetylase Pta, which is required for acetate production (Fig. 1A). Viability of this deletion strain indicated that metabolites (like acetyl-phosphate) produced downstream of Pta are not required for growth under the tested static fermentation condition. In aerated cultures, biomass yield did not change compared to that of static growth, but the *pta* mutant produced ~8-fold less acetate than the WT while acetoin levels were slightly increased (Table 2). In contrast, Pta was required for aerobic respiration, as the biomass yield was intermediate between those observed in the WT growing under aerated and respiration growth. Although the medium pH values were similar between strains (Table 2), the amount of lactate increased in the mutant compared to that of the WT strain (8.5 versus 0.2 mM/OD_{600} for mutant and WT, respectively). We conclude that the acetate pathway contributes mainly to biomass yield rather than to pH homeostasis under aerobic respiration.

Acetoin pathway contributes to pH homeostasis. Interruption of the *als* gene to block acetoin production (Fig. 1A) had little effect on fermentation (static or aeration) growth, as determined by OD_{600}, medium pH, and metabolic patterns (Table 2). In contrast, the *als* mutant was disabled for aerobic respiration, and its biomass yield and medium pH values were much lower than those of the WT (Table 2). The decrease of medium pH value to 4.8 in the *als* mutant (compared to ~6.2 in the WT strain) correlates with a concomitant increase in the amount of lactate (16 versus ~0.2 mM/OD_{600} for mutant and WT, respectively). Amounts of acetate were slightly higher in the mutant (Table 2). The *als* mutant was complemented by a plasmid-carried *als*

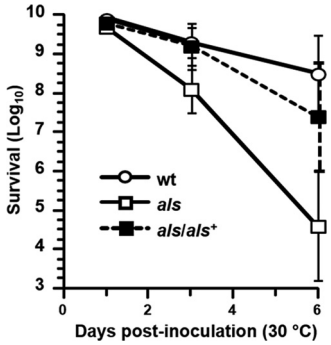

FIG 3 *als* deletion decreases survival capacity of *L. lactis* MG1363 under respiration growth conditions. Strains (WT, *als* mutant, and its *als* mutant/*als*$^+$ complemented strain) were grown under respiration conditions in M17 glucose at 30°C. Aliquots were removed at different times for counting. Data and standard deviations are presented and are from at least three independent experiments.

gene expressed from its own promoter. Complementation restored a profile close to that of the WT, thereby confirming that the phenotype is due to the *als* deletion. Interestingly, addition of sodium hydroxide (40 mM to maintain pH at 6.2) restored ~70% of the biomass gain by the *als* mutant due to respiration metabolism (Table 2). Altogether, the results indicate that the acetoin pathway participates extensively in pH homeostasis, which directly affects the physiology of respiration metabolism. Nevertheless, our data indicate that *als* inactivation does not abolish respiration growth, as observed in the *pta* deletion.

The acetoin pathway is essential for long-term survival. Acidification of growth medium has a negative impact on *L. lactis* survival (5, 11). As the *als* mutant acidified the medium (Table 2), its mortality in stationary phase would be expected to be higher than that of the WT strain. As expected, the *als* mutant showed a strong decrease in survival under respiration conditions compared to that of the WT strain (Fig. 3). Six days postinoculation at 30°C, *als* mutant survival was less than 10^5 CFU/ml, while WT counts were ~10^9 CFU/ml (5, 11). Complementation with the cloned *als* gene restored the survival capacity of the *als* mutant in respiration (Fig. 3). Notably, in static or aerobic fermentation growth, the *als* mutant and the WT strain showed similar mortality curves (data not shown). In contrast, the *pta* mutant, which displayed pH values close to that of the WT strain under respiration (Table 2), survived like the WT strain (Fig. S2). We conclude that the acetoin pathway is required for good survival of cells grown under respiration conditions, likely for maintaining pH homeostasis.

***als* expression is linked to perturbation of pyruvate catabolism.** As the acetoin pathway is connected to pH homeostasis, we suspected that *als* expression responds to an increase of pyruvate (or pyruvic acid) in the cell. Aeration and respiration growth did indeed produce acetoin and acetate and expression of pyruvate accumulation, and *als* expression should be more marked under respiration than under aeration growth. To test our hypothesis, we cloned the promoter region of the monocistronic *als* (600 bp) upstream of the promoterless *lacLM* genes encoding β-galactosidase in a plasmid derivative of pAK80 (18), giving P_{als}-*lacLM*_pBC629.2 (chloramphenicol [Cm]; see Materials and Methods). The plasmid was established in strain MG1363, and *als* expression was compared between cells grown under fermentation and respiration conditions (Fig. 4A). Under static conditions, we observed a basal level of *als* expression. As expected, expression increased >2-fold up to 5-fold during aerobic fermentation and respiration, respectively. We repeated assays in *als* and *pta* mutants grown under respiration growth and found that *als* expression increased much earlier in the *als* mutant than in other strains. Under aeration, *als* expression was slightly higher in the *als* mutant than in the others, while no differences were observed between strains grown under static conditions (Fig. S3). As respiration decreased acidification (Table 2) (5, 8), we asked whether a decrease of pH affected *als* expression. The β-galactosidase

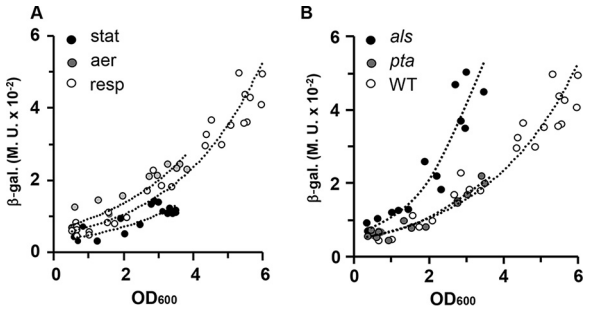

FIG 4 *als* expression in *L. lactis* strains growing under different growth conditions. (A) Wild-type strain, carrying plasmid P_{als}-*lacLM*_pBC629.2, was grown under static (black circles), aeration (gray circles), and respiration (white circles) conditions in M17 glucose at 30°C. Data correspond to superposition of three independent experiments with their respective trend curves. (B) *als* (black circles), *pta* (gray circles) mutants, and the wild-type strain (white circles), carrying the plasmid P_{als}-*lacLM*_pBC629.2, were grown under respiration growth conditions. At different times, aliquots were harvested for β-galactosidase determinations. β-Galactosidase is expressed in specific activity (M. U.; as described for Fig. 2). Data correspond to superposition of three independent experiments with their respective trend curves. Values for the wild-type strain are given in panel A.

activities were measured in cells growing in M17 glucose at different initial pH values (under aerobic fermentation) (Fig. S4). In the range of pH values between 7 and 6, no major differences were observed between these conditions. However, under a value of 5, *als* expression increased 4-fold versus that at pH 7 and in the exponential growth phase. Altogether, our observations suggest that accumulation of pyruvate, rather than acidification, is a signal for *als* expression.

Metabolic shift is a common behavior in *L. lactis* species growing under respiration conditions. In *L. lactis* strain MG1363 or CHCC2682, respiration displayed a drastic metabolic shift, with production of acetate and acetoin at the expense of lactic acid. To determine whether this shift occurs in other *L. lactis* strains, we compared growth parameters (OD_{600} and pH) and end product patterns between cells growing under aerated fermentation and respiration conditions. As shown in Table 3, respiration growth conditions changed end product patterns, as expected. Moreover, the metabolic shift improved gain of biomass yield at diverse ranges. In strains IL1403 and CHCC2871, the gains were the lowest. We can conclude that the metabolic shift is a general behavior of respiration in *L. lactis* species.

DISCUSSION

We previously showed that *L. lactis* activates respiration metabolism and provokes major metabolic changes that improve bacterial growth and greatly prolong survival (5, 8, 11). Using a global approach, we identified factors that may be responsible for *L. lactis* reprogramming during respiration growth, in particular genes involved in pyruvate catabolism when cells attain stationary phase. Studies of specific mutants lead us to propose that the acetate pathway plays a role in biomass yield, whereas the acetoin pathway is involved in maintenance of metabolic activities by avoiding internal acidification. The balance between these two pathways may thus be key parameters for optimizing *L. lactis* growth and survival.

The present study reveals that respiration dramatically altered gene expression when cells reach stationary phase, in contrast to exponential phase, when expression changes were limited (8). Late expression changes affect genes involved in pyruvate catabolism, including *pdhABCD*, *ackA*, and *adhE*. Interestingly, these genes belong to a regulon governed by CcpA, the global regulator of carbon catabolite repression (19). CcpA responds to both the nature and amounts of available carbohydrates, leading us to suggest that the observed expression changes are due to carbohydrate depletion. However, CcpA is not the only regulator of pyruvate catabolism (19). As an example, in the acetoin pathway, we observed induction of *als* and *aldB* (Table 1), whereas in acetate we found *pflB*. From our data, we suspect that the pool of pyruvate (rather than

TABLE 3 Metabolic shift, a specific behavior of respiration growth in *L. lactis* species[a]

Strain and condition	OD_{600}	pH	Level of[b]: Lactate	Acetate	Acetoin
IL1403					
A	2.48 ± 0.1	5 ± 0.0	29.5 ± 2.1	4.5 ± 0.4	3.5 ± 0.1
R	3.14 ± 0.4	5.54 ± 0.4	18.3 ± 5.2	2.6 ± 0.6	6.4 ± 1.9
CHCC5915					
A	3.16 ± 0.0	4.85 ± 0.0	27.9 ± 3.3	2.7 ± 0.0	2.1 ± 0.0
R	4.89 ± 0.1	5.63 ± 0.1	10.3 ± 1.2	3.5 ± 0.1	5.4 ± 1.3
CHCC6005					
A	2.26 ± 0.2	5.72 ± 0.1	18.1 ± 2.5	5.8 ± 2.0	1.7 ± 0.3
R	4.92 ± 0.2	5.89 ± 0.0	6.7 ± 0.7	3.6 ± 0.2	8 ± 2.3
CHCC2862					
A	2.96 ± 0.0	4.82 ± 0.0	30.6 ± 0.4	2.2 ± 0.4	2.3 ± 0.1
R	4.27 ± 0.0	5.83 ± 0.1	12.2 ± 1	2.4 ± 0.2	7.8 ± 1.5
CHCC2871					
A	2.32 ± 0.0	4.57 ± 0.0	45.8 ± 7	0.1 ± 0.0	1.6 ± 0.1
R	2.59 ± 0.1	5.92 ± 0.1	19.8 ± 0.3	1.0 ± 0.2	14.1 ± 2.5
CHCC4436					
A	3.08 ± 0.1	4.83 ± 0.0	30.4 ± 3.2	2.6 ± 0.3	1.8 ± 0.1
R	4.78 ± 0.1	5.57 ± 0.0	9.9 ± 1.1	3.9 ± 0.4	5 ± 1.1

[a]Strains are *L. lactis* subsp. *lactis* (IL1403, CHCC2682, CHCC2871, and CHCC6005) or subsp. *cremoris* (CHCC4436 and CHCC5915). A, aeration; R, respiration.

[b]Metabolite levels were determined from overnight culture and expressed in mM/OD_{600}. Means of data represent averages from at least two independent experiments.

pyruvic acid) controls the acetoin pathway. Deletion of the chromosomal *als* compared to that of *pta*, for instance, led to increased reporter-driven *als* expression (Fig. 4B). In some organisms, like *Bacillus subtilis*, *als* expression is governed by a LysR-type regulator, AlsR; *als* and *alsR* are colocalized (20). While no Lys-R type regulator was found close to *als* in *L. lactis*, one of the regulators induced during respiration growth (Table 1) may be a candidate to regulate this pathway.

The acetate pathway drives biomass yield. Biomass yield depends at least in part on the bacterial capacity to produce ATP. Our results indicate that respiration growth optimizes ATP availability by both economizing spending and increasing its production. In *L. lactis*, the ATP pool comes from glycolysis. Part of this pool is consumed by the ATPase for H^+ extrusion during fermentation growth. However, once respiration is activated by heme supplementation, *L. lactis* replaces the ATPase as an H^+ extrusion pump by the respiratory chain (9), thus saving ATP expenditure and making ATP more available. In this context, ATPase could also reverse its activity by producing ATP from reentry of H^+ extruded by the respiratory chain. However, analysis of ATP production suggested that this reaction is poorly efficient (21). The acetate kinase is thus a likely source of ATP (22, 23), as *pta* (Table 2) and *ack* (not shown) mutants show low biomass under respiration growth conditions. To produce acetyl-phosphate, *L. lactis* MG1363 requires both PDC and, to a lesser extent, PflB. While PDC activity is expected under aerobiosis, it is remarkable that PflB, which is an oxygen-sensitive enzyme, contributes to biomass yield under aerobic respiration (Table 2). PflB activity is likely attributed to the protective effect of the respiratory chain against oxidative stress (11). We conclude that the acetate pathway is needed to benefit from respiration growth for improved biomass.

The acetoin pathway drives pH homeostasis. Low pH is responsible for *L. lactis* growth arrest in sugar-rich media and is a known cause of bacterial mortality (5, 24). Several mechanisms of acid stress tolerance in *L. lactis* have been described and involve expression of ATPase, the arginine deiminase pathway, or purine depletion (24).

The present work gives evidence that respiration also improves cell survival by rerouting pathways that impact pH. First, pyruvic acid, which is highly acidic (pK_a

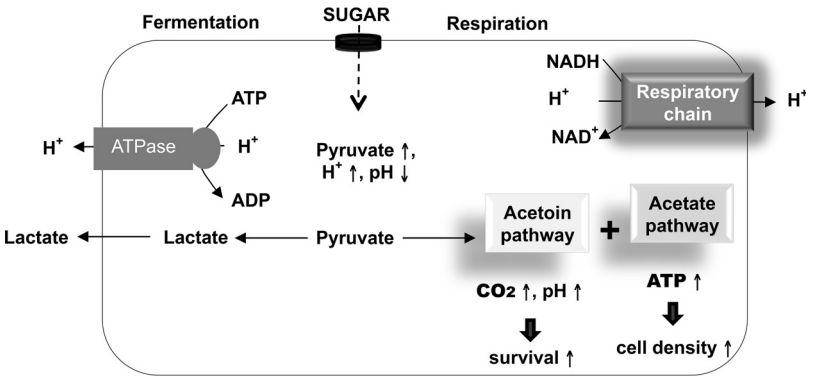

FIG 5 Role of pyruvate catabolism in *L. lactis* under respiration growth conditions. Under fermentation, the pyruvic acid pool, coming from glycolytic activity, splits into pyruvate and protons. Lactate dehydrogenase reduces pyruvate in lactate, while ATPase extrudes protons. Under respiration growth conditions, the respiratory chain consumes NADH, which hampers Ldh activity, which requires NADH. The pyruvate and proton pool thus increases and triggers a drop of internal pH. To prevent acidification, cells convert pyruvate to acetoin and CO_2. Together these changes allow cells to maintain their metabolic activity (gain in growth and survival). A proportion of pyruvate is also used to produce acetate, thereby generating ATP (gain in biomass yield). The two main pathways expressed in respiration growth are highlighted by shading.

around 2.4), is shunted via Als toward production of acetoin, which is neutral. Second, CO_2, a by-product of acetolactate decarboxylase activity (likely AldB) in the acetoin pathway, further contributes to pH homeostasis by neutralizing acidic compounds. Note that PDC activity also provides CO_2 from pyruvate (Fig. 1). Remarkably, all genes in the acetoin pathway are upregulated in respiration growth, and *als* deletion leads to a pH reduction and striking drop in survival (Table 1 and Fig. 3). Moreover, neutralizing the medium with NaOH was sufficient to restore almost complete respiration activity (Table 2). Our findings indicate that the acetoin pathway protects cell robustness by preventing bacterial acidification and may thus be responsible for the greatly improved stability associated with respiration growth (5).

Based on results of the present study, we propose the following metabolic model (Fig. 5). During growth, cells first convert carbohydrate into pyruvate (and its acidic form) via glycolytic activity. The large metabolite pool leads to a decrease in internal pH. Under fermentation, pyruvate is reduced to lactate and is secreted, while ATPase extrudes protons. During respiration, the respiratory chain replaces ATPase for proton extrusion. *L. lactis* induces an acid stress response, with conversion of pyruvate into neutral compounds via the acetoin pathway. This proposed strategy would contribute to maintaining activity of acid-sensitive enzymes. Thus, cells are well prepared for long-term survival once they attain stationary phase. Concomitantly, the pyruvate pool also fuels the acetate pathway for ATP production via acetate kinase and extends growth.

MATERIALS AND METHODS

Growth conditions. *L. lactis* strains (Table 4) were grown in reconstituted M17 broth supplemented with sugars (glucose 1% and lactose 1% for MG1363 and CHCC2862, respectively) and antibiotics when required. Cultures were inoculated at an OD_{600} of 0.025 in fresh medium and incubated under static conditions, aeration conditions (culture volume is 1/10 of flasks; agitation at 200 rpm), or respiration (aeration plus 2 to 5 μM heme [Sigma]). Cultures were incubated at 30°C and harvested at the specified cell densities. *Escherichia coli* strain TG1 was used as the recipient strain for cloning. Antibiotics used were the following: in *L. lactis*, erythromycin (Ery), 1 μg ml^{-1}; tetracycline (Tet), 3 μg ml^{-1}; chloramphenicol (Cm), 5 μg ml^{-1}; in *E. coli*, erythromycin, 150 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}. Antibiotic concentrations follow the Clinical and Laboratory Standards Institute guidelines.

Microarray analysis. RNA purification and microarrays were performed as described by Pedersen et al. (8).

Mutant and plasmid constructions. Transcriptional fusions were constructed to measure promoter activities and expressed from the chromosome or on a plasmid (Table 4). Vector pAK80, carrying the promoterless *lacLM* coding for β-galactosidase, was used for both constructions. In the first case the *lacLM* operon was purified from plasmid pAK80 after digestion with XmaI and SalI and then ligated into

TABLE 4 Strains and plasmids

Strain, plasmid, or primer	Description or sequence	Source or reference
L. lactis		
CHCC2862	*L. lactis* subsp. *lactis*, lactose$^+$	8
CHCC2871	*L. lactis* subsp. *lactis*, lactose$^+$	Chr. Hansen A/S
CHCC4436	*L. lactis* subsp. *cremoris*, lactose$^+$	Chr. Hansen A/S
CHCC5915	*L. lactis* subsp. *cremoris*, lactose$^+$	Chr. Hansen A/S
CHCC6005	*L. lactis* subsp. *lactis*, lactose$^+$	Chr. Hansen A/S
IL1403	*L. lactis* subsp. *lactis*	Our laboratory
MG1363	Plasmid free, *L. lactis* subsp. *cremoris*	28
pflB mutant	MG1363, insertion of pRV300 into *pflB* gene	This study
pdhA mutant	MG1363, insertion of pRV300 into *pdhA* gene	This study
als mutant	MG1363, insertion of pRV300 into *als* gene	This study
pta mutant	MG1363, deletion of *pta* gene	This study
E. coli		
TG1	K-12 *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5, (r_k^- m_k^-) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	
Plasmids		
pRV300	Amp_{Ecoli}, $Ery_{Llactis}$	26
pAK80	Promoterless *lacLM*, Ery	Eric Johansen, Chr. Hansen A/S
pBC629.2	pBR322 derivative, with pCT1138 replicon $Cm_{Llactis}$	Laboratory, 29
pBR322-pGhost8	Amp_{Ecoli}, $Tet_{Llactis}$	P. Gaudu
pBC570.1	pRV300-*lacLM*, Amp_{Ecoli}, $Ery_{Llactis}$	This study
pGhost3 Cm		25
P_{rcfB}-pAK80		14
Primers		
Transcriptional fusion		
pflB (llmg_0629) on pBC570.1		
P_pflB_For	GCGGCCGCTTCTCACCAGGTGCTAACCC (NotI)	
P_pflB_Rev	CCCGGGTTAGATATTTGAAGTGTGCATTACTTCTTCATC (XmaI)	
pfl_extFor	GAAGATCTTACAGAAACCAAGATTCAGC	
poxL (llmg_2321) on pBC570.1		
P_poxL-For	GATCGCGGCCGCGCTTTGCCGGCAGCAATTGG (NotI)	
P_poxL-Rev	GATCCCCGGGTTATTCTTTAATAAGTTTACTG (XmaI)	
poxL_extFor	AAAGATTTACGACAAGTGG	
pbuX (llmg_1345) on pBC570.1		
P_pbuX_For	GATCGCGGCCGCCAGGATTTTCACAAAACGTTGG (NotI)	
P_pbuX_Rev	GATCCCCGGGTTATTTTGCTTCATCAGCAGCTAATTCATCTTTTTTTCC (XmaI)	
pbuX_extFor	AAGTTGTTGGTCTAGGAATGG	
dtpT (llmg_1865) on pBC570.1		
P_dtpT_For	GATCGCGGCCGCAAAGTTCTACGATTATTGCG (NotI)	
P_dtpT_Rev	GATCCCCGGGTTAACGAACATCTCCCATTAATTTC (XmaI)	
dtpT_extFor	TTCTGCTATTGTCTTTTGGG	
msmK (llmg_0446) on pBC570.1		
P_msmK_For	GATCGCGGCCGCGCAATGACACTTGCTGACCG (NotI)	
P_msmK_Rev	GATCCCCGGGTTAATCAACGATACGGTGTTCAG (XmaI)	
msmK_extFor	AACAACCATTTACGTTACCC	
butBA (llmg_1641-1642) on pBC570.1		
P_butBA_For	GATCGCGGCCGCCCGATTGAAACAATTACTCC (NotI)	
P_butBA_Rev	GATCCCCGGGTTAATGAAATTGCATTCCAC (XmaI)	
butBA_extFor	GTAGTAGTAAATAATGCAGG	
LacLM-Rev	ATTGAGACTCTTGATGAAGCG	
als (llmg_1309) on pAK80		
P_als_For	GATCAGATCTTGAAACTGTCTTTCTACTTGC (BglII)	
P_als_Rev	GATCGGATCCTTCATATTTCATAATCTCTTGG (BamHI)	
Mutants		
pflB (llmg_0629)		
M_pfl_For	GATCCCGCGGGAATTCGATAATCTTTGAACAAGCTTGGG (SacII)	
M_pfl_Rev	GATCACTAGTGATTAGCAAGACGTGCATATACCC (SpeI)	
pdhA (llmg_0074)		
M_pdhA_For	GATCTGCAGTATCACTGCAAGACACCG (PstI)	
M_pdhA_Rev	TTAGGTACCTTAGGCTTTATAGGCACCAGC (KpnI)	
pdhA_Ext-For	TCTTAGATTTCAAAACGC	

(Continued on next page)

TABLE 4 (Continued)

Strain, plasmid, or primer	Description or sequence	Source or reference
als (llmg_1309)		
M_als_For	GATCTGCAGTCGATAGTTTGATTAACC (PstI)	
M_als_Rev	TTAGGTACCTTATCCAACCAAAATTACTGGC (KpnI)	
als_Ext-For	TCTGAGAAACAATTTGGG	
pta (llmg_1642) deletion		
M_pta_For	GATCCTCGAGGCTCTCAAGAAACTTACCGCC (XhoI)	
M_pta_intRev	CTGACAGAATTTCAATCTATTATTCCATTTTAACAATATCCCTCG	
M_pta_intFor	CGAGGGATATTGTTAAAATGGAATAATAGATTGAAATTCTGTCAG	
M_pta_Rev	GATCAAGCTTAGCTAGAATGAAACAAATGGC (HindIII)	
pta_For_ext	GCTTCAATACGGACGTGCGG	
pta_Rev_ext	CAATGCATTCAACCATTGGGG	
als complementation in pGhost3		
C_als$^+$_For	GGATTTTATGCGTGAGAATGTTACTGATGAAACTGTCTTTCTACTTGC	
C_als$^+$_Rev	GGTAACTATTGCCGGGATAGACTCAACTATCATTCTAAATTTCACC	

the sites of suicide plasmid pRV300 to generate plasmid pBC570.1. We then amplified by PCR a fragment corresponding to the 3′ end of the open reading frame of *pflB*, *poxL*, *pbuX*, *dtpT*, and *msmK* (including the stop but excluding the terminator) with appropriate primer pairs and the genomic DNA of MG1363 as a template. The PCR product was cloned into the pBC570.1 multicloning site (MCS) (Table 4). The resulting plasmid was established in *E. coli* strain TG1 and selected on Amp. *L. lactis* strain MG1363 was then transformed with the plasmid using Ery for selection. Plasmid in the gene locus was confirmed by PCR with one primer in the gene (ext) and a second in the *lacLM* gene (Table 4). Plasmid-expressed promoter fusions were similarly constructed with appropriate primer pairs (*butBA* and *als*) and ligated into the MCS of pAK80. To monitor *als* expression in the *als* mutant background (Ery-resistant strain), the P_{als}-pAK80 plasmid was digested by BamHI and SalI and cloned into pBC629.2. P_{als}_*lacLM*_pBC629.2 plasmid was established in *E. coli* and then *L. lactis* strains on plates supplemented with appropriate antibiotics. All PCR products were confirmed by sequencing.

For the *pta* deletion mutant construction we proceeded as follows. Two DNA fragments covering the upstream and downstream regions of the *pta* gene (Table 4) were PCR amplified and then fused by a second PCR. The resultant fragment was ligated to pBR322-pGhost8 digested with XhoI and HindIII. The modified plasmid was established in *E. coli* strain TG1 and transferred to *L. lactis* strain MG1363. Transformants were selected on Tet at 30°C. Plasmid integration in the locus and excision were performed as described previously (25). Deletion of *pta* was confirmed by PCR with primer pairs chosen outside the recombination region. *als*, *pflB*, and *pdhA* genes were inactivated by single cross-over recombination that truncated >60% of open reading frames by insertion of the suicide plasmid pRV300 (26). A DNA fragment of 500 bp, corresponding to the 5′ part of the gene, was ligated into the MCS of pRV300. The modified plasmids were first established in *E. coli* strain TG1 on Amp selection and then transferred to *L. lactis* strain MG1363 on Ery selection. In-locus integration was confirmed by PCR.

To confirm that *pflB* inactivation was responsible for the observed phenotypes, we grew the *pflB* mutant (Ery-resistant strain) in the absence of antibiotics to select for plasmid excision and *pflB* restoration. A *pflB* revertant was chosen for phenotypic analysis (Table 2). The *als* mutant was complemented with a plasmid (pGhost3, Cm) carrying the *als* gene expressed from its own promoter.

Survival assays. Strains were grown at 30°C under respiration conditions, and cell suspensions were removed at various times for plating on nonselective M17 glucose-agar medium.

***β*-Galactosidase assays.** Cells were grown to the appropriate OD_{600}. Aliquots were collected, centrifuged, and recovered in fresh Z buffer. Cells were permeabilized by adding SDS and $CHCl_3$ at 0.0025% and 5%, respectively. *β*-Galactosidase assays were performed as described previously (27) and expressed as specific activity using Miller units (M. U.).

Accession number(s). Array data are available at NCBI GEO under accession number GSE56776.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01005-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We have no conflicting interests to declare.

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